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Abstract: Zebrafish (Danio rerio) larvae have been suggested as vertebrate model to complement or even replace mammals for rapidly assessing behavioral effects of psychoactive drugs. Yet, divergent responses have been reported in mammals and fish despite the conservation of many drug targets. Cocaine, eg, acts as stimulant in mammals but no such response has been documented for zebrafish larvae. We hypothesized that differences in exposure routes (inhalation or injection in mammals vs waterborne in fish) may be a reason for differences in behavioral responses. We characterized cocaine toxicokinetics by liquid chromatography-mass spectrometry and found its rapid uptake into larvae. We used Matrix-assisted laser desorption ionization-mass spectrometry imaging for the first time to characterize internal distribution of cocaine in zebrafish larvae. Surprisingly, eyes accumulated the highest amount of cocaine and retained most of it even after 48 h depuration. We attribute this to trapping by pigment melanin, a thus far little explored mechanism that may also be relevant for other basic drugs. Cocaine also reached the brain but with levels similar to those in trunk indicating simple passive diffusion as means of distribution which was supported by toxicokinetic models. Although brain levels covered those known to cause hyperactivity in mammals, only hypoactivity (decreased locomotion) was recorded in zebrafish larvae. Our results therefore point to cocaine's anesthetic properties as the dominant mechanism of interaction in the fish: upon entry through the fish skin and gills, it first acts on peripheral nerves rapidly overriding any potential stimulatory response in the brain.

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Zebrafish Larvae Are Insensitive to Stimulation by Cocaine: Importance of Exposure Route and Toxicokinetics

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ABSTRACT

Zebrafish (*Danio rerio*) larvae have been suggested as vertebrate model to complement or even replace mammals for rapidly assessing behavioral effects of psychoactive drugs. Yet, divergent responses have been reported in mammals and fish despite the conservation of many drug targets. Cocaine, eg, acts as stimulant in mammals but no such response has been documented for zebrafish larvae. We hypothesized that differences in exposure routes (inhalation or injection in mammals vs waterborne in fish) may be a reason for differences in behavioral responses. We characterized cocaine toxicokinetics by liquid chromatography-mass spectrometry and found its rapid uptake into larvae. We used Matrix-assisted laser desorption ionization-mass spectrometry imaging for the first time to characterize internal distribution of cocaine in zebrafish larvae. Surprisingly, eyes accumulated the highest amount of cocaine and retained most of it even after 48 h depuration. We attribute this to trapping by pigment melanin, a thus far little explored mechanism that may also be relevant for other basic drugs. Cocaine also reached the brain but with levels similar to those in trunk indicating simple passive diffusion as means of distribution which was supported by toxicokinetic models. Although brain levels covered those known to cause hyperactivity in mammals, only hypoactivity (decreased locomotion) was recorded in zebrafish larvae. Our results therefore point to cocaine's anesthetic properties as the dominant mechanism of interaction in the fish: upon entry through the fish skin and gills, it first acts on peripheral nerves rapidly overriding any potential stimulatory response in the brain.

Key words: cocaine; zebrafish; locomotor activity; distribution; LC-MS/MS and MALDI imaging.

The steadily increasing number of psychoactive substances entering the market, both licit and illicit, is intensifying the demand to assess their safety and efficacy. Current assessment methods rely on rodents such as mice and rats and hence require substantial cost and time investments. This limits the risk assessment to a few substances per year. According to the

European Drug Record 2015 (João Goulão, 2015), only 6 such substances were tested in 2014; this being regarded as an unprecedented achievement. In contrast, over 400 illicit psychoactive drugs entered the market since 2005—with 101 new substances in 2014 alone. Therefore, scientists and regulators alike agree that new approaches to behavioral testing are needed. These should be fast and ideally less controversial in terms of animal ethics.

One suggested alternative approach to behavioral testing in rodents is the use of zebrafish (*Danio rerio*). Indeed, as a vertebrate, zebrafish has conserved pharmacological targets and nervous system structures comparable with mammals, including humans (Howe *et al.*, 2013; Rinkwitz *et al.*, 2011). When compared with rodents, zebrafish offers advantages such as higher fecundity, faster development, and lower quantities of test chemical required (Banote *et al.*, 2013; Rihel and Ghosh, 2015). Especially the larval stage of zebrafish offers features attractive for drug screening: the larvae can be housed in multiwell plates, thus not only requiring little volumes for testing but also offering the potential for medium to high-throughput screening of diverse endpoints, including behavioral alterations (Kokel *et al.*, 2010; Rihel *et al.*, 2010). On the other hand, the small size of the larvae has thus far prevented that observed drug effects were linked to concentrations at target sites, even though the importance of this aspect has been highlighted (Rihel and Ghosh, 2015).

Despite the widespread advocacy for the use of zebrafish as a general vertebrate model for behavioral studies (Best *et al.*, 2008; Winter *et al.*, 2008), our understanding of the neurological and pharmacological bases of behavior in this species remains rather limited. Moreover, the concordance of neurobehavioral effects of chemicals between zebrafish and mammals is relatively low, especially for larval stages. For example, cocaine, a classical drug with known stimulant properties in mammals (Benowitz, 1993; Degenhardt and Hall, 2012), produced only hypoactive responses in zebrafish larvae (Irons *et al.*, 2010). The reasons for this discrepancy are not yet understood.

Cocaine acts on monoaminergic neurotransmitter systems (Ritz *et al.*, 1987). Lower comparability of behavioral responses between larval zebrafish and adult mammals may be due to the incomplete maturation of neural circuits in the former. Another important difference between these models may be the exposure route: while in mammals cocaine and other psychoactive drugs are usually inhaled, snorted or injected (Booze *et al.*, 1997), they are commonly delivered by waterborne exposure in zebrafish (Boehmler *et al.*, 2007; Irons *et al.*, 2010). This may result in large differences in uptake and internal distribution, ie, pharmaco- or toxicokinetic processes, which in turn may influence the effects observed. For example, apart from its stimulatory actions in the brain, cocaine is known to also act as a local anesthetic by blocking voltage-gated sodium channels. This effect is particularly relevant for peripheral nerves (Dunwiddie *et al.*, 1988; Ikemoto and Witkin, 2003). Thus, it can be hypothesized that the effects elicited by cocaine may differ depending on the internal distribution patterns and concentrations at particular target sites.

In this study, we aimed to explore if the peculiarities of cocaine toxicokinetics in zebrafish larvae exposed through water may explain the discrepancy in the behavioral effects observed in zebrafish larvae and mammals. We focused explicitly on larvae because this life stage is widely used to test psychoactive drugs. We approached our research question by simultaneously assessing behavioral effects and toxicokinetic profiles of cocaine. Uptake, biotransformation and elimination were studied by liquid-chromatography-tandem mass spectrometry (LC-MS/MS) in conjunction with toxicokinetic modeling, while drug distribution was characterized by matrix assisted laser desorption and ionization mass spectrometry imaging (MALDI-MSI). Our work shows that employment of different exposure routes results in significant differences in toxicokinetics, likely bearing a significant contribution to the opposing behavioral responses to cocaine observed in zebrafish larvae and mammals.

MATERIALS AND METHODS

Experimental Animals

Wild-type zebrafish originally obtained from OBI petshop (Leipzig, Germany) were maintained and bred in our facility according to the guidelines published by (Nüsslein-Volhard and Dahm, 2002). Fish were housed in groups of 25–30 and were reared in static containers in reconstituted water [294.0 mg/l $CaCl_2.2H_2O$, 123.2 mg/l $MgSO_4.7H2O$, 64.74 mg/l $NaHCO_3$ and 5.7 mg/l KCl; ISO 15088:2007(E); 2007]. Manual water exchange and siphoning of solid wastes was carried out once a week. Fish were fed twice daily with a combination of live food (*Artemia nauplia*) and dry flakes (Tetramin, Switzerland). The room was maintained at 28°C and 14/10 h light/dark cycle. Eggs for exposure studies were obtained by group crosses and were collected 1 h after the lights were turned on. They were washed and raised in embryo medium in petri dishes of approximately 50 per dish until 5 days post-fertilization (dpf) in an incubator at 28°C with the same light/dark cycle conditions as mentioned earlier. All procedures were in accordance with the animal protection guidelines. Experiments with the larvae were approved by the Cantonal Veterinary Office.

Uptake, Biotransformation, Distribution and Elimination Kinetics

Uptake, Biotransformation and Elimination of Cocaine

Cocaine hydrochloride was purchased from Lipomed AG, Switzerland. For exposures of larvae, 1 mM stock solution of cocaine was dissolved in an appropriate volume of embryo medium to achieve the desired concentrations.

To identify the range of non-toxic to toxic concentrations of cocaine, zebrafish larvae were exposed at 4 dpf for 24 h to cocaine at concentrations of 5, 50, and 500 μM. The larvae were monitored on 5 dpf and the abnormalities were noted. Based on the results obtained from this study, all further experimentation was limited up to 50 μM (Supplementary Figure S1).

Larvae of 5 dpf were exposed to 5 and 50 μM cocaine. For the uptake analysis, 16 larvae were pooled into pre-weighed 2 ml lysing matrix tubes containing metal beads (MP Biomedicals, France), after 0.25, 0.5, 1, 3, 6, 8, 10, 24, 27, and 31 h exposure, washed with PBS and frozen in liquid nitrogen. To study the clearance of cocaine from larvae, they were transferred into cocaine-free medium (reconstituted water medium) after 24 h of uptake and collected at 1, 3, 8, 24, 27, 31, and 48 h of depuration following the same procedure. Exposure medium was also collected during uptake and depuration experiments at the same time points and preserved until further analysis by freezing in liquid nitrogen.

On the day of analysis, fish samples were defrosted first at 4°C for 1 h and then at room temperature for 2 h. A volume of 1 ml of ammonium formate buffer (5 μM, pH 3.1) was added and samples were homogenized in Fast Prep-24 Instrument (MP Biomedicals, France). The homogenate was spiked with internal

standard at a concentration of 5 μM cocaine-D_3 and 0.25 μM norcocaine-D_3 (cocaine metabolite) and extracted with acetonitrile (Sigma Aldrich, Switzerland). After centrifugation for 5 min at 10 000 rpm, the supernatant was mixed with eluent C: D (eluent C: 25 mM ammonium acetate pH 4 and eluent D: acetonitrile with 0.1% acetic acid) at a ratio of 1:1 and was analyzed for cocaine and its metabolites benzoylecgonine (BEC), ecgonine methyl ester (EME) and norcocaine using LC-MS/MS. The calibration curves were linear in the concentration ranges of 0.05–20 μM for cocaine and 0.0025–3 μM for norcocaine. For analysis of exposure medium samples, they were diluted 1000 times with eluent and internal standards were added at the same concentrations as described earlier before the LC-MS/MS analysis.

Analytes were separated on a Kinetex PFP (pentafluorophenyl) liquid chromatography (LC) column (50 × 2.1 mm, 2.6 μm) maintained at 35 °C. Detection was performed by Applied Biosystems 5500 Q Trap linear ion trap (LIT) triple quadrupole mass spectrometer (AB Sciex, Germany) with Analyst software (Version 1.5.1, AB Sciex, Germany). The mobile phase gradient was applied at a flow rate of 0.35 ml/min with eluent C and eluent D. The gradient was programmed as follows: 0–0.5 min: 10% eluent D; 0.5–4.5 min: gradient increase to 90% eluent D; 4.5–5 min: 90% eluent D; 5–5.010 min: gradient decrease to 10% eluent D; 5.010–6 min: 10% eluent D. Multiple reaction monitoring (MRM) transitions were acquired for cocaine (304 → 182), norcocaine (290 → 168), BEC (290 → 168), and EME (200 → 182). Cocaine and norcocaine concentrations were quantified based on the standard curves. Due to the non-enzymatic formation, BEC and EME were also detected in the exposure medium, therefore these 2 metabolites were not followed further in internal concentration measurements.

Distribution of Cocaine: Visualisation by MALDI MSI

This work is the first report where MALDI imaging analysis was performed in zebrafish larvae. Distribution analysis of cocaine in the whole body was performed after 8 h of exposure by matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI MSI). Larvae were euthanized by cold water euthanasia followed by 3 washes in PBS. Individual larvae were positioned on blocks containing Optimal Cutting Temperature media (Thermo Scientific, USA) and frozen by placing them on dry ice. Transverse, sagittal and coronal sections of 16 μm thicknesses were made with the cryotome (Microm HM 560, Thermo Scientific, USA) and optical images were taken with the Leica microscope (Leica Application Suite Advanced Fluorescence, Mannheim, Germany).

MALDI matrix (2, 5 dihydroxybenzoic acid) was applied using a robotic matrix application system (TM-sprayer, HTX Technologies, USA). MALDI-MS images were acquired in MRM mode with positive ionization on a Flashquant Workstation (AB Sciex, Germany) fitted with a MALDI source consisting of a high repetition laser (Nd:YAG, 355 nm; 1000 Hz). The laser had an elliptic shape of 200 × 100 μm and worked in the continuous rastering oversampling mode leading to a spatial resolution of 50 μm. For image formation, the intensity of the MRM signals was translated into a false colour scale with black representing low and red high cocaine concentrations. Enhanced product ion (EPI) spectra for confirmation were acquired in discrete mode with a laser burn time of 1 s and a scan speed of 1000 amu/s applying dynamic LIT fill time. Data were acquired with Analyst 1.4.2 Software (AB Sciex). Raw data conversion from wiff files to image files was performed with dedicated software provided by Markus Stoeckli (Novartis, Basel) and AB Sciex. Tissue view Software (v1.1, AB Sciex) was used for image processing. Subsequent to mass spectrometry imaging, sections were stained with Hematoxylin Ehrlich (TCS Biosciences, UK) and Shandon Eosin Y (Thermo Scientific, USA) according to the procedure recommended by the supplier (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). To locate the spatial distribution of cocaine, MALDI images were overlaid with the optical images. The current theoretical spatial resolution of our method (50 μm) does not allow a detailed characterization of the neuroanatomy of the brain and eye regions showing cocaine signals. In the future, MALDI imaging method in zebrafish larvae should be further optimized to achieve a better spatial resolution allowing a more precise description of internal distribution patterns.

Distribution of Cocaine: Quantification in Dissected Larval Tissues

Larval brain, eyes and trunk were dissected out as described elsewhere (Turner *et al.*, 2014), at 2 time points, 1 after 8 h of exposure to cocaine and another 1 following 48 h of depuration period. Briefly, larvae were anesthetized in 0.02% tricaine (Sigma-Aldrich) and moved to a Sylgard glass petri dish. Under zoom stereo microscope (Leica, Germany), eyes were detached gently from the head and brains were dissected out using sharp tungsten needles (Fine Science Tools, Germany) and forceps (Dumont no. 5 superfine, Fine Science Tools, Germany). Brains, eyes and trunks were pooled from 16 fish into different tubes, snap frozen in liquid nitrogen and stored at −80 °C until analysis. Samples were analyzed using LC-MS/MS with the method described above for the kinetic samples analysis and quantified against their respective matrix standard curves.

Toxicokinetic Modeling

One-Compartment Approach

One-compartment models were chosen to describe and predict internal concentrations of cocaine in the whole zebrafish larvae as well as to determine empirical uptake and elimination constant rates for cocaine. This approach assumes that a chemical which enters an organism is distributed instantaneously and equally. This concept can be described by the following equation (Landrum *et al.*, 1992; Stadnicka *et al.*, 2012):

$$\frac{d}{dt}C_{int}(t) = k_{in} \cdot C_w(t) - k_{out} \cdot C_{int}(t) \quad (1)$$

where $C_{int}(t)$ is the internal chemical concentration (amount × mass^{-1}), $C_w(t)$ is the chemical concentration in the water (amount × volume^{-1}), k_{in} is the uptake rate constant (volume × mass^{-1} × time^{-1}) and k_{out} is the elimination rate constant (time^{-1}).

Empirical uptake and elimination constant rates for cocaine were determined by calibrating respective parameters from equation 1 based on measured data of cocaine concentrations in water and larvae over time, obtained in this study.

Measured internal concentrations of cocaine were compared with predicted values obtained by using a simple 1-compartment toxicokinetic model (Hendriks *et al.*, 2001). This approach describes the accumulation kinetics of organic chemicals as a function of the octanol-water partition coefficient, as well as the lipid content, weight, and trophic level of the species.

Multi-Compartment Approach

In order to account for biotransformation processes and chemical distribution in the larvae, a multi-compartment concept was implemented. Here, internal concentration of cocaine (ie,

parental compound) was described over time by the following equation:

$$\frac{d}{dt}C_{int\_cocaine}(t) = k_{in} \cdot C_w(t) - \left(k_{out} + \sum k_{biotr\_met\_i}\right) \cdot C_{int\_cocaine} \quad (2)$$

where $C_{int\_cocaine}(t)$ is the internal concentration of cocaine (amount × mass^{-1}), $C_w(t)$ is the cocaine concentration in the water (amount × volume^{-1}), k_{in} is the uptake rate constant (volume × mass^{-1} × time^{-1}), k_{out} is the elimination rate constant (time^{-1}) and $k_{biotr\_met\_i}$ is the biotransformation rate to metabolite i (time^{-1}).

Internal concentration of each metabolite can then be described by the following equation:

$$\frac{d}{dt}C_{int\_met\_i}(t) = k_{in\_met\_i} \cdot C_{w\_met\_i}(t) + k_{biotr\_met\_i} \cdot C_{int\_cocaine} - k_{out\_met\_i} \cdot C_{int\_met\_i} \quad (3)$$

where $C_{int\_met\_i}(t)$ is the internal concentration of metabolite i (amount × mass^{-1}), $C_{w\_met\_i}(t)$ is the concentration of metabolite i in the water (amount × volume^{-1}), $k_{in\_met\_i}$ is the uptake rate constant (volume × mass^{-1} × time^{-1}), $k_{out\_met\_i}$ is the elimination rate constant (time^{-1}), $k_{biotr\_met\_i}$ is the biotransformation rate to metabolite i (time^{-1}) and $C_{int\_cocaine}(t)$ is the internal concentration of cocaine (amount × mass^{-1}).

In order to describe chemical distribution in the larvae, eyes were distinguished as a separate compartment and treated independently to other larvae's tissues in the way that different constant rates were determined for the eyes and for the rest of the body. The relationship between chemical concentration in eyes and in the whole fish was determined over time by the following equation:

$$C_{int\_larvae}(t) = \frac{C_{int\_eyes}(t) \cdot W_{eyes} + C_{int\_rest}(t) \cdot W_{rest}}{W_{eyes} + W_{rest}} \quad (4)$$

where $C_{int\_larvae}(t)$ is the chemical concentration in the whole larvae (amount × mass^{-1}), $C_{int\_eyes}(t)$ is the chemical concentration in the larvae's eyes (amount × mass^{-1}), $C_{int\_rest}(t)$ is the chemical concentration in the larvae's tissues and organs other than eyes (amount × mass^{-1}), W_{eyes} is the weight of eyes (mass) and W_{rest} is the weight of larvae's tissues and organs other than eyes (mass). Larvae's weight is the sum of W_{eyes} and W_{rest}.

Model Implementation and Calibration

All models were implemented and solved using ModelMaker (version 4.0, Cherwell Scientific Ltd., Oxford, UK) with the settings described next.

1. Run
   - start value: 0,
   - stop value: last day of exposure,
   - repeated run: no.
2. Integration
   - random seed: 1,
   - integration method: Runge-Kutta,
   - output points: user defined (dependent on stop value)
   - fixed step: no,
   - accuracy: 10^{-6},
   - minimum value: 10^{-10},
   - approx. no of steps: 100,
   - error scaling: a constant value (10).
3. Calibration
   - Optimization run:
     - Method: Marquardt,
     - Weighting: ordinary least squares.
   - Optimization Settings:
     - Convergence Change: 0.1,
     - Convergence Steps: 50,
     - Retry Count: 50,
   - Marquardt settings:
     - Initial Lambda: 100,
     - Minimum Change: 1e-200,
     - Fractional Change: 0.01.

Locomotor Behavior Assessment

Larvae at 5 dpf were distributed 1 larvae per well in 500 µl volume in a 48-well plate in the morning and were returned to the housing incubator for 3–4 h for acclimatization. Locomotor activity was recorded using the ZebraLab™ behavior tracking system (Version 3, ViewPoint, Lyon, France) that has a camera frame rate of 25 frames per second. The software assessed the duration of the movements and distances traveled that were categorized in the user interface into 3 different movements: low speed movements (≤2 mm/s), medium speed movements (2–10 mm/s) and high speed movements (≥10 mm/s). Detection threshold was set to 20 for tracking the animal excluding the background.

After acclimatization, larvae were challenged with 5, 10, 15, 25, and 50 µM of cocaine. Locomotor activity was assessed at 3 time points; first 1 being acute exposure, where plate was placed on the recording stage in dark immediately after adding the drug and tracking was started after 20 min of drug exposure. Recording was carried out for 70 min with dark and light phases alternating each 10 min. Individual larva locomotion was quantified and assessed as distance traveled (mm) every 2 min. Second time point was after 8 h exposure to cocaine; recording was carried out using the same protocol as for acute exposure. This time point was chosen because the uptake analysis showed that the plateau phase was reached around 8 h of exposure. Finally, locomotor activity was also analysed after 48 h of excretion phase (for the 50 µM concentration only). This time point was chosen because in the depuration experiment we observed that the drug was retained by the fish eye even after 48 h of depuration, allowing to address the question whether cocaine in the eye affects locomotory behavior in zebrafish larvae.

Statistical Analysis

All the data were exported to and analyzed using Microsoft Excel 2010. All graphs were plotted in GraphPad Prism® (Version 6 for Windows, GraphPad Software, San Diego, California, USA). For the behavior assessment, statistical analysis was performed using 'RStudio' (Version 0.98.1103, USA) by repeated measures Analysis of Variance (ANOVA). Data were first assessed for the significance of independent factors, ie, time, concentration and plate with the dependent factor, ie, the locomotor activity. Data were then segregated based on the light and dark conditions (considering from the first light condition that followed dark period) and ANOVA was performed with lighting conditions and drug treatment as independent factors and the distance traveled as the dependent factor to determine whether the concentration has an effect on the lighting conditions. Finally, at each lighting condition, ANOVA was performed comparing each

concentration to control to see if there is a significant effect; drug treatment being independent and distance traveled being a dependent factor followed by Bonferroni's *post-hoc* analysis (Supplementary Table S2). Statistical significance was set at $\alpha = 0.003$.

RESULTS AND DISCUSSION

Cocaine is Rapidly Taken Up by Zebrafish Larvae

To test if cocaine is taken up by the zebrafish larvae from the exposure medium and to understand the kinetics of uptake, we exposed larvae to 5 μM (1.5 mg/l) and 50 μM (15 mg/l) of cocaine and quantified it over time in both the medium and the fish (Supplementary Table S1A). A 5 μM cocaine concentration was previously demonstrated to be the lowest concentration causing a behavioral response (ie, hypoactivity) in zebrafish larvae upon waterborne exposure (Irons *et al.*, 2010). The 50 μM cocaine concentration was used as the highest one not causing an impact on zebrafish larvae survival and morphology (Supplementary Figure S1). Cocaine was quantifiable in zebrafish larvae whole-body homogenates from the first time point on (15 min) and its concentration continued to increase with an uptake rate constant, k_{in}, of 12.9 l·kg^{-1}·h^{-1} up to about 8 h, resulting in cocaine internal concentrations of 81 and 445 mg/kg for the 5 and 50 μM exposures, respectively (Figure 1). The uptake rate constant for cocaine is in a range similar to that reported previously in 3–5 days post fertilization (dpf) zebrafish for 3 synthetic pyrethroids (6.6–20.3 l kg^{-1} h^{1}) (Tu *et al.*, 2014). The pyrethroids have 3 orders of magnitude higher octanol-water partition coefficient (logKow), suggesting a limited role of this physico-chemical parameter in terms of chemical uptake rates.

Being a consequence of waterborne exposure route used in zebrafish, a continuous increase of internal cocaine concentrations during several hours sharply contrasts to the uptake profiles common in mammals. In latter, the frequently applied snorting, inhalation, intravenous, or subcutaneous injection routes represent a short-term exposure and yield a fast delivery into the blood stream, reaching mean peak plasma concentrations in 5–30 min (Kolbrich *et al.*, 2006; Mendelson *et al.*, 1998; Perez-Reyes *et al.*, 1994).

Cocaine is Only Slowly Eliminated from Zebrafish Larvae

Upon transfer to clean water, zebrafish larvae eliminated about 50% of cocaine within 8 h and about 63% within 24 h. Thereafter, little further elimination occurred so that after 48 h of elimination, about 30% of the cocaine still remained in the fish (Figure 1 and Supplementary Table S1B). A biphasic pattern of elimination from adult zebrafish was reported for different anilines and nitrobenzenes, with a much slower second elimination rate and retention of 10–20% for anilines and 50% for dinitrobenzene (Kalsch *et al.*, 1991). The authors concluded that the 2-phase elimination indicates processes other than passive diffusion and speculated that a particular spatial distribution could have led to only partial elimination. In humans, cocaine is quickly eliminated at least from plasma (Kolbrich *et al.*, 2006).

Zebrafish Larvae Accumulate Much More Cocaine than Predicted Based on Whole Body Passive Diffusion

We next applied a simple 1-compartment toxicokinetic model (Hendriks *et al.*, 2001; Stadnicka *et al.*, 2012), which assumes passive uptake and elimination of cocaine based on its pH-corrected octanol-water partition coefficient (logD = 1.22). When doing so, we observed that the measured internal concentrations of cocaine were much higher than the predicted values (Figure 1). These results are indicative of either a sink in the fish body and/or an active uptake mechanism. In support of the latter, Chapy *et al.* (2015) demonstrated that cocaine is predominantly taken up by a proton-antiporter across the blood-brain barrier in rodents. Elimination of cocaine from the zebrafish larvae also deviated significantly from the predictions by the toxicokinetic model. We therefore hypothesized that (1) cocaine could be present at locally increased concentrations in distinct compartments of the fish body and (2) fish retain the cocaine in such a distinct compartment from which it cannot easily be removed.

Cocaine Concentrates Predominantly in the Fish Eye

After an exposure to 50 μM cocaine for 8 h, ie, the time needed to reach maximum internal concentrations, coronal sections of the larvae were prepared and analyzed by MALDI-MSI. The cocaine signals revealed accumulation in the head region and in

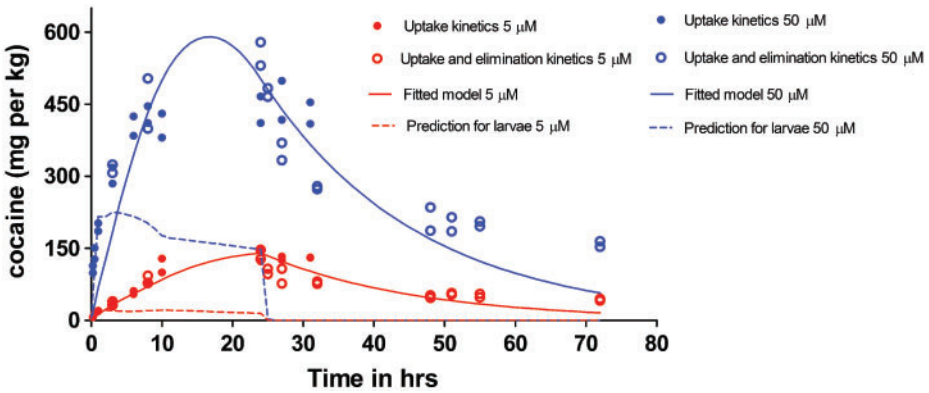

FIG. 1. Uptake and elimination kinetics of cocaine in zebrafish larvae. Zebrafish larvae were exposed starting at 5 dpf for 24 h and thereafter placed in cocaine-free medium for up to 48 h. Cocaine was quantified in whole body homogenates by LC-MS/MS. Filled dots—data from independent experimental replicates that assessed uptake only; empty dots—data from independent experimental replicates that assessed both uptake and elimination. In red—uptake and elimination kinetics for a starting exposure concentration of 5 μM cocaine; in blue——uptake and elimination kinetics for a starting exposure concentration of 50 μM cocaine. Solid line—fitted 1-compartment model; dashed line—prediction based on our physiologically based toxicokinetic model.

the trunk (Figs. 2A and B). To locate the cocaine in the head region more precisely, we prepared sagittal and transverse sections. The former showed cocaine accumulation in the eyes (Figs. 2C and D), while the latter indicated the presence of cocaine in the forebrain (Figs. 2E and F), midbrain (Figs. 2G and H), and hindbrain (Figs. 2I and J). The signal that was detected in the MALDI MS was also confirmed by acquiring the corresponding EPI spectrum for cocaine and comparison with the reference spectrum (Supplementary Figure S2).

To confirm the qualitative results obtained by MALDI-MSI, we also quantified cocaine by LC-MS/MS in dissected brain, eyes and trunk after 8 h exposure to 50 μM cocaine. Strikingly, by far the highest concentration of cocaine was found in the eyes (1516 ± 84 mg/kg), followed by the brain (406 ± 44 mg/kg) and the trunk (255 ± 32 mg/kg) (Figure 3A). The similarity of cocaine concentrations in the brain and the trunk suggests that a functional blood-brain barrier is lacking at this stage, what is in line with an earlier study that found an incomplete development of the blood brain barrier in zebrafish larvae up to 9–10 dpf (Jeong *et al.*, 2008). Interestingly, much lower cocaine levels in the brain were associated with cocaine intoxication leading to death in humans (0.17–31 mg/kg; Spiehler and Reed, 1985) and mice (4 mg/kg; McCarthy *et al.*, 2004). It has to be noted though that the majority of cocaine fatalities have been attributed to cardiovascular complications rather than to any neurological effects. We also measured cocaine in the dissected brain, eyes, and trunk after 24 h exposure followed by 48 h elimination. In this scenario, the concentration of cocaine in the zebrafish eye (1331 ± 98 mg/kg) was still remarkably similar to that measured after 8 h, while the concentrations in brain (50 ± 3.7 mg/kg) and trunk (44 ± 2.2 mg/kg) had dropped to very low levels (Figure 3B). This shows that cocaine not only accumulates in the fish eye to a very high concentration but that it is also strongly retained by this tissue.

Norcocaine, a Biotransformation Product of Cocaine, Likewise Concentrates in the Fish Eye

To find out whether cocaine reaches the eye by internal distribution in the zebrafish larvae or via diffusion directly from the exterior exposure medium, we analyzed the concentrations of norcocaine in the dissected tissues (Figure 3C). Norcocaine, BEC, and EME are the biotransformation products of cocaine. The former metabolite can be produced only enzymatically while the latter 2 can also be formed by non-enzymatic degradation (Supplementary Figure S3) (Jufer *et al.*, 1998; Warner and Norman, 2000). The distribution pattern of norcocaine was very similar to that of cocaine. We assume that cocaine undergoes N-demethylation to produce norcocaine mostly in the liver, like in mammals (Brittebo, 1988; Inaba *et al.*, 1978; Jufer *et al.*, 1998). Therefore, the distribution pattern of norcocaine suggests that cocaine reached the eye through internal distribution rather than directly from the exterior. However, in mammals it has been shown that the pigmented epithelium of the eye contains drug metabolising enzyme activity (Shichi *et al.*, 1975; Zanger and Schwab, 2013), although the expression levels particularly of CYP3A4 were very low compared with the liver (Zhang *et al.*, 2008). In zebrafish larvae, CYP3A65 (shared synteny with CYP3A4 in mammals) was shown to be highly expressed in liver and intestine, and very low in the eye (Saad *et al.*, 2016; Tseng *et al.*, 2005). Hence, we cannot completely rule out that a very low amount of cocaine could have been taken directly from the exposure medium through the eyes and biotransformed there directly. The norcocaine concentration in the analyzed body parts after 8 h of cocaine exposure was about 0.6% compared with that of cocaine, while in the whole larvae it varied from 0.5 to 3.6% at different time points which compares well to 3% reported in humans (Jufer *et al.*, 1998; Kolbrich *et al.*, 2006).

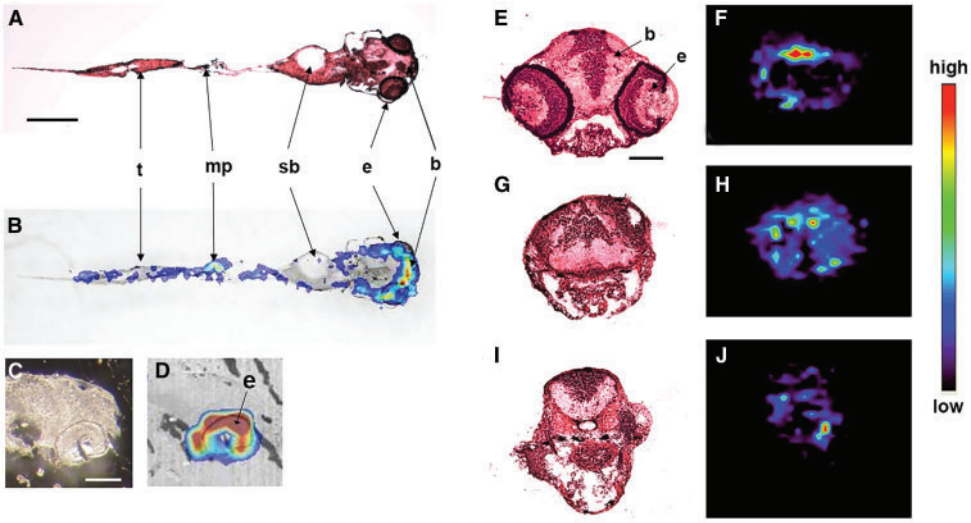

FIG. 2. Cocaine distribution in zebrafish larvae at 5 dpf exposed to 50 μM cocaine for 8 h visualized by MALDI imaging. Coronal sections are depicted as Hematoxylin & Eosin (H & E) stained optical image **(A)** and overlay of an optical image with the MALDI image **(B)**, indicating cocaine distribution in the trunk and in the head region, ie, in the brain and/or eyes, scale bar: 300 μm. Sagittal sections with optical image **(C)** and overlay of an optical image with the MALDI image **(D)** confirm the accumulation of cocaine in the eye, scale bar: 150 μm. Transverse sections made across the head region through forebrain (H & E-stained optical image—**E** and overlay of an optical image with the MALDI image—**F**), midbrain (H & E stained optical image—**G**, overlay of an optical image with the MALDI image—**H**) and hind brain (H & E-stained optical image—**I**, overlay of an optical image with the MALDI image—**J**) confirm the presence of cocaine in the brain, scale bar: 100 μm. Abbreviations: b, brain; e, eye; sb, swim bladder; mp, melanophores; and t, trunk. It is important to note that the optical images of transverse sections were not superimposed with the corresponding MALDI images but are presented next to each other. It was not achievable to overlay them due to the small size of the tissue and resolution limits of the instrument. Untreated control fish showed no cocaine signal.

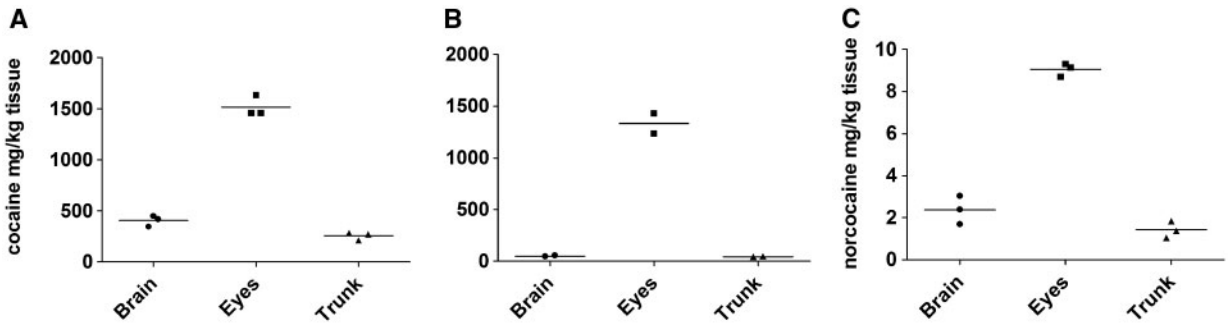

FIG. 3. Concentrations of cocaine and norcocaine quantified by LC-MS/MS in dissected tissues. Zebrafish larvae at 5 dpf were exposed to 50 μM of cocaine for 8 h **(A, C)** or exposed to 50 μM of cocaine for 24 h followed by 48 h of elimination **(B)** before brain, eyes and trunk were dissected and analyzed for cocaine (A, B) and norcocaine (C). Note that the y-axis scales for cocaine and norcocaine differ. Values are reported as original data (symbols; each data point was obtained from tissues pooled from 16 larvae) and mean of 3 independent experiments for uptake measurements (A, C—horizontal line) and 2 independent experiments for the elimination (B - horizontal line). For the uptake measurements (A, C), 1-way ANOVA followed by Tukey's multiple comparison test revealed significant difference for brain versus eyes and trunk versus eyes (both $P < .0001$), while no statistical difference was seen for brain versus trunk [$P = .0894$ (A) and 0.112 (C)].

Cocaine and Norcocaine Follow Simple Partitioning in the Brain and Trunk but Not in the Eye

Based on the observed cocaine and norcocaine distribution, we developed a multi-compartment toxicokinetic model to distinguish the eye compartment from the rest of the body (ie, brain and trunk) and compared the measured and the predicted uptake and elimination kinetics (Figs. 4A and B). Results demonstrate that for both chemicals the distribution in the body compartment without the eyes (brain and trunk) follows the assumption of simple partitioning whereas in the eyes the kinetics of cocaine and norcocaine is distinct.

Taken together, we conclude that, following waterborne exposure, cocaine is distributed not only to the trunk and the brain but much more so to the eyes, where it can remain for prolonged times. In fact, accumulation of drugs in the eyes of cats, dogs, rabbits, and mice after systemic administration has been observed already several decades ago for diverse psychopharmacological agents, such as phenothiazines and amitriptyline and for anti-malarial drugs like chloroquine and amopyroquine (Mason, 1977). For zebrafish larvae, high accumulation of organic forms of mercury, namely methylmercury chloride and methylmercury L-cysteineate, in the eyes, in particular the outer layer of the lens, has been reported (Korbas *et al.*, 2012).

One reason for the high concentration of cocaine and norcocaine in the eyes could be their basic and lipophilic properties, which might lead to their entrapment in melanophores due to a high affinity to melanin (Hubbard *et al.*, 2000; Nakahara *et al.*, 1995). Transfer of an electron from a donor substance to melanin has been discussed to result in a trapping mechanism strong enough to account for slow dissociation of melanin-drug complexes (Mason *et al.*, 1960). Interestingly, our MALDI images suggested that in the trunk, cocaine is associated with specific structures which resemble melanophores (Figs. 2A and B). High affinity to melanin would also explain the slow clearance from the eye, a phenomenon that has previously been described for ophthalmic drugs in humans (Al-Ghananeem and Crooks, 2007) and for intravenously injected chlorpromazine in rabbits (Potts, 1962). Future studies on the bioaccumulation of psychoactive drugs or other chemicals in zebrafish should thus take the eye as a potential site of local entrapment and increased concentration into account.

Having characterized the accumulation and distribution of cocaine in zebrafish larvae, we next studied its effects on locomotory behavior in different exposure scenarios, aiming to understand the correlation between toxicokinetic aspects and the behavioral alterations observed.

Zebrafish Larvae Respond to Cocaine with Hypo- but Not Hyperactivity

We exposed larvae in 3 ways prior to analysis of locomotion: as conventionally done for short-term exposure (20 min) (Figure 5A; see also Irons *et al.*, 2010), after 8 h of uptake (Figure 5B) and after 24 h of uptake followed by 48 h of elimination (Figure 5C). Locomotion in zebrafish larvae is characterized by higher activity in the dark compared to light. In agreement with the results presented in Irons *et al.* (2010), zebrafish larvae showed a concentration-dependent decrease in their overall locomotor activity (hypo-activity) upon short-term exposure to cocaine (Figure 5A). Hypoactivity was more severe in the dark than in the light, with statistically significant effects observed at concentrations of 5–10 μM and higher (Supplementary Table S2). Zebrafish larvae exposed to cocaine concentrations below 5 μM did not show significant differences compared with control (Supplementary Figure S4). In the larvae exposed to cocaine for 8 h, a similar behavioral pattern was observed, albeit with greater concentration-dependent differences in amplitude (Figure 5B), likely explained by higher internal concentrations of cocaine accumulated by this time (Figure 1). At this time point, high concentrations of cocaine were observed in the eyes but also in the brain and trunk (Figure 3A).

After 48 h of elimination, the behavior of exposed larvae became indistinguishable from that of control animals (Figure 5C). Since at this time point high cocaine concentrations were still observed in the eye but not in the brain or trunk, we can conclude that (1) observed hypoactivity is caused by cocaine accumulated either in the brain or the trunk or both and (2) cocaine accumulation in the eye does not affect locomotory behavior in zebrafish larvae. However, cocaine was demonstrated to cause a decrease in visual sensitivity in adult zebrafish (Darland and Dowling, 2001). Thus, future research should investigate if other functions associated with vision could be affected in the larvae as well.

Possible Reasons for the Lack of Hyperactivity

In all behavioral experiments performed, only hypoactive responses to cocaine were observed even though a wide range

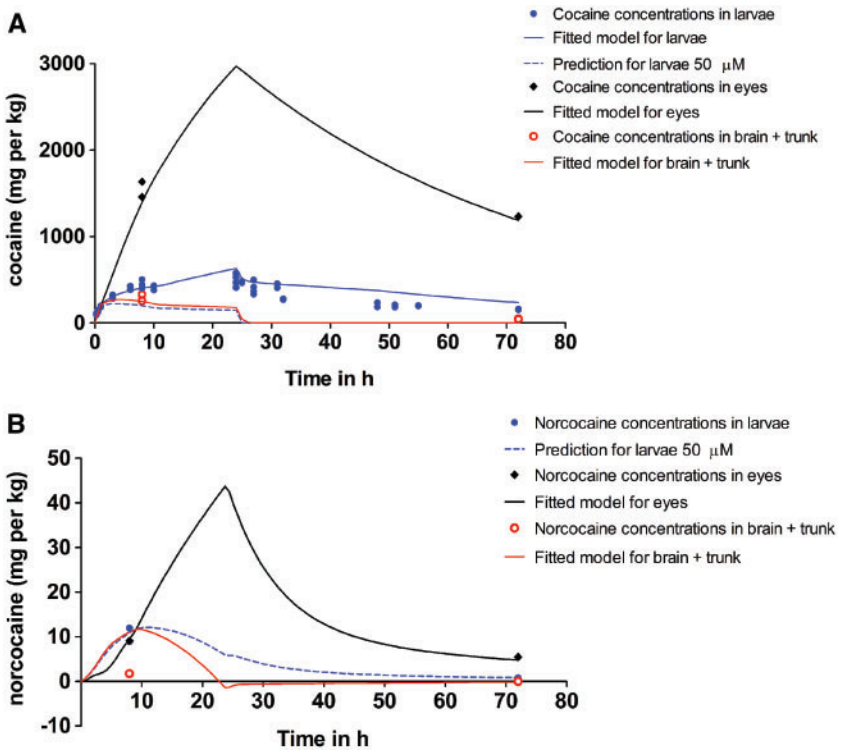

FIG. 4. Uptake and elimination kinetics modeling when eyes and rest of the body (ie, brain and trunk) are treated as 2 separate compartments. Zebrafish larvae were exposed to 50 μM cocaine for 24 h starting at 5 dpf and thereafter placed in cocaine-free medium for up to 48 h. Cocaine **(A)** and norcocaine **(B)** were quantified by LC-MS/MS in whole body homogenates and in dissected brain, eyes and trunk. In blue—uptake and elimination kinetics for whole larvae; in red—fitted multi-compartment model for brain and trunk together; in black—fitted multi-compartment model for the eyes. Multi-compartment model fit shows that distribution in the brain and trunk follows model predictions based on simple partitioning while distribution in the eyes is distinct.

of exposure concentrations and different exposure scenarios were assessed. Reports on the behavioral effects of acute exposure to cocaine in rodents show hyperactivity (Antoniou *et al.*, 1998; Badanich *et al.*, 2008; Niculescu *et al.*, 2005) and a dose related stimulation, with a maximal stimulation of the locomotor activity at a dose of 59 μmol/kg (≈17 mg/kg) (Katz *et al.*, 1999). Zebrafish larvae exposures resulting in comparable total body concentrations of 6 mg/kg (5 μM, 15 min exposure) and about 100 mg/kg (50 μM, 15 min exposure) showed only hypoactivity while lower exposure concentrations had no measurable effect on locomotion. Moreover, as discussed above, brain tissue concentration in zebrafish larvae were even higher than cocaine levels in human brain reported to be lethal. Thus, neither total organism nor brain tissue concentrations can explain the difference in behavior. We postulate 3 reasons for the lack of hyperactivity in zebrafish larvae. The first one refers to potential differences in the zebrafish catecholaminergic system compared to mammals. The functional homologs of the midbrain dopaminergic system, being one of the targets of cocaine, are not well established in zebrafish (Panula *et al.*, 2010; Yamamoto and Vernier, 2011). The second possible explanation is an interaction of cocaine with serotonin pathway. Such an interaction has been demonstrated to lead to hypoactive response in *Caenorhabditis elegans* (Ward *et al.*, 2009). Yet, one important observation suggests to draw off attention from the brain as a sole active target site. This observation relates to the fact that concentrations in the brain were similar to those in the trunk, ie, no brain-specific cocaine accumulation occurred. Therefore we propose that it is the local anesthetic property of cocaine that causes the strong suppression of locomotor activity observed in zebrafish (Butterworth and Strichartz, 1990; Dunwiddie *et al.*, 1988; Ikemoto and Witkin, 2003). Because the surface of the fish larvae is quite large and because all of the cocaine first passes through the gill and the skin as barriers, the local anesthetic action in peripheral nerves, more specifically blockage of voltage-gated sodium channels in muscle and gill (Chopra *et al.*, 2007; Dunwiddie *et al.*, 1988; Ikemoto and Witkin, 2003), becomes dominant even before cocaine can act in the brain.

To conclude, in search for the cause of the differences in locomotory response to cocaine in zebrafish larvae and mammals, we demonstrate the importance of taking toxicokinetic processes into account. We show that, while waterborne exposure does not restrain the uptake of cocaine by the organism, the kinetics of uptake is very different from that established via snorting, inhalation or injection routes in mammals. High internal concentrations accumulated by zebrafish larvae suggest its overall lower sensitivity to cocaine intoxication compared to mammals. Cocaine concentrations in brain and trunk were similar to each other, indicating a lack of functional blood-brain barrier at this stage. Surprisingly, the largest accumulation of cocaine was found in the zebrafish eye, which we demonstrate for the first time by combining LC-MS/MS analysis and MALDI imaging. Entrapment and slow elimination of cocaine could be a feature of melanin-drug complexes, which has been suggested decades ago. An existence of specific retention sites within the organism was also suggested by our toxicokinetic modeling. This raises the question of the role of melanin in the accumulation and slow elimination of drugs and other chemicals in

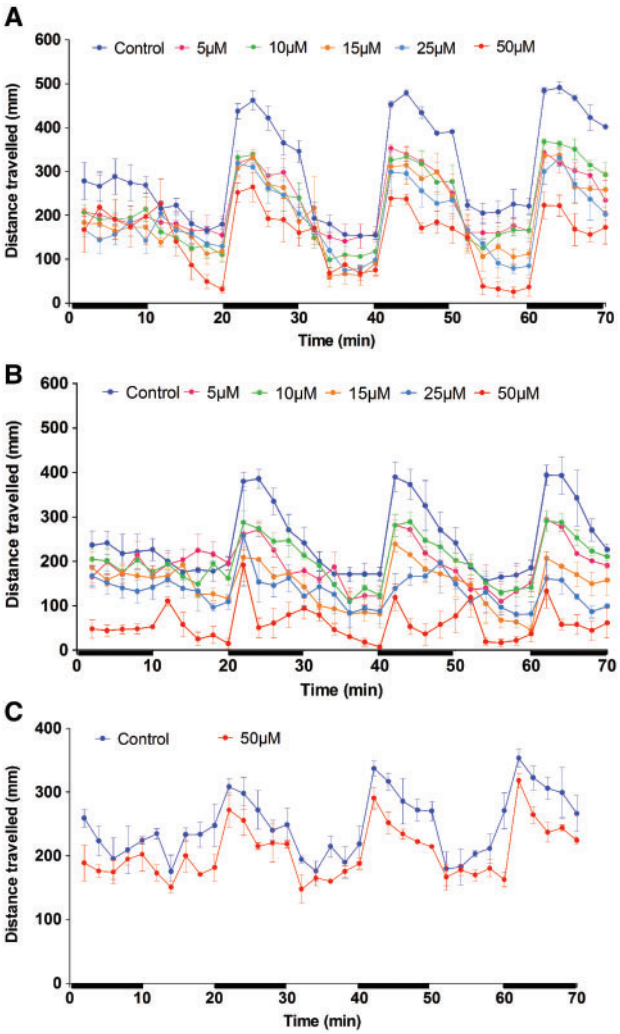

FIG. 5. Locomotor activity of zebrafish larvae treated with cocaine. At 5 dpf larvae were exposed to different concentrations of cocaine for 20 min **(A)**, 8 h **(B)**, or 24 h followed by 48 h of depuration **(C)**. Distance traveled by the fish was analyzed every 2 min for 70 min with dark (black bars) and light phases alternating every 10 min. Data were assessed using repeated measures ANOVA. Data segregated and analyzed for the effect of concentration at different lighting conditions revealed significant effect of cocaine on the locomotory activity after 20 min of exposure (A) in both light ($P = 6.34e\text{-}07$) and dark phases ($P = 3.25e\text{-}12$) and after 8 h of exposure (B) in both light ($P \leq 2e\text{-}16$) and dark phases ($P \leq 2e\text{-}16$). Locomotor activity measured after 24 h of exposure followed by transfer into clean water for 48 h (C) shows no significant effect. Values are reported as mean ± SEM from 3 independent experiments ie, 3 plates (n = 8 larvae/concentration/plate).

zebrafish larvae in general. Finally, because of the fast and significant uptake of cocaine, the fish solely respond with hypo- but never with hyperactivity. We propose that the permeation of the cocaine through the fish surface upon the waterborne exposure leads to anesthetic interactions with peripheral nerves and overrides any potential hyperactive response that could be evoked from interaction with stimulatory targets in the brain.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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